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Interaction of rat lung SSAO with the novel 1-N-substituted thiocarbamoyl-3-substituted phenyl-5-(2-pyrolyl)-2-pyrazoline derivatives

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Summary Interactions of twelve new synthesized 1-N-substituted thiocarbamoyl-3-substituted phenyl-5-pyrolyl-2-pyrazoline derivatives with rat lung semicarbazide-sensitive amine oxidase (SSAO) were assessed. Pyrazoline derivatives were synthesized according to previous methods and SSAO was purified from the crude microsomal fractions of rat lung.

Three compounds (3e, 3f, 3k) with a *p*-methoxy group at the phenyl ring inhibited rat lung SSAO non-competitively and irreversibly, and showed higher affinity towards SSAO when expressed in terms of IC_{50} for SSAO/Monoamine oxidase B (MAO-B). Since these novel pyrazoline derivatives have been found to act as suicide inhibitors of SSAO, the semicarbazide group in these molecules may be responsible for the SSAO inhibitory action. It is suggested that these compounds cannot enter the first small active site cavity of SSAO and may interact tightly with another binding site or with some other reactive groups present in the molecule. Compound 3e showed the highest inhibitory activity on rat lung SSAO. The novel pyrazoline derivatives may be used to discriminate between Cu- and FAD-containing amine oxidases and may have promising features as anti-Parkinson agents if the SSAO-inhibitory effects can be supported by *in vivo* studies.

Keywords: Semicarbazide-sensitive amine oxidase (SSAO), rat lung, purification, inhibition, pyrazolines

Introduction

Semicarbazide-sensitive amine oxidases (SSAO, E.C. 1.4.3.6.) are a group of enzymes containing copper and quinone. Most SSAOs are dimeric glycoproteins with molecular masses of 140–180 kDa (Salminen et al., 1998). It has been postulated that the enzyme may be involved in detoxifying xenobiotics, regulating glucose uptake, and effecting cell adhesion, leukocyte trafficking and angiogenesis (Boomsma et al., 2000; Ferrer et al., 2002). Increased plasma SSAO activities were reported in alcoholics, and in patients with diabetes, Alzheimer's disease, Parkinson's disease and heart and vascular diseases (Boomsma et al., 1997; Ucar and Demir, 2003; Yu and Zuo, 1997). Thus, the design and synthesis of new compounds as specific SSAO inhibitors may be useful for discriminating between MAO and SSAO and for developing novel therapeutic agents.

On the basis of our previous studies, which have shown that some pyrazoline derivatives containing a thienyl ring had MAO inhibitory activities (Gokhan et al., 2003), the present study aimed to purify SSAO from rat lung and to evaluate the newly synthesized pyrazoline derivatives as novel SSAO inhibitors.

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Material and methods

Preparation of rat lung microsomes

SSAO was purified from rat lung (Ethics Committee of Laboratory Animals in Hacettepe University, Turkey, 2004/36, 1082) according to the method described by Lizcano et al. (1994) with some modifications. The lung tissue was homogenized 1:10 (w/v) in 20 mM Tris-HCl buffer, pH 7.2 containing 0.25 M sucrose and centrifuged at $10{,}000 \times g$ for 10 min. After centrifuging the supernatant at the same velocity, the pellet was discarded and 10 mM $CaCl_2$ was added to the supernatant and centrifuged at $25{,}000 \times g$ for 30 min. This was resuspended in 20 mM potassium phosphate buffer, pH 7.2 containing 150 mM KCl and centrifuged at $25{,}000 \times g$ for 30 min. The final pellets, resuspended in 20 mM potassium phosphate buffer, pH 7.2, and stored at $-80°C$, are referred to as crude microsomes.

Solubilization of rat lung microsomes

Solubilized enzyme was obtained from the crude microsomes by a method previously described (Lizcano et al., 1998). The crude microsomal fraction was mixed with an equal volume of 1% Triton X-100 (w/v) in 20 mM potassium phosphate buffer, pH 7.2, and the mixture was stirred for 30 min at 4°C. The solubilized enzyme was obtained by decanting the supernatant after centrifugation at $105{,}000 \times g$ for 1 h and was stored at −80°C.

Purification of solubilized rat lung microsomes

The solubilized enzyme was loaded onto a Cibacron Blue 3GA-agarose (18 ml) column, previously equilibrated with 0.1% (w/v) Triton X-100 in 20 mM potassium phosphate buffer, pH 7.2 (buffer A). The resin was washed out with an ample amount of equilibration buffer and, after passing 20 ml of 0.5 M KCl in buffer A, the SSAO activity was eluted with 40 ml of 1 M KCl in buffer A. Fractions (0.8 ml) containing SSAO activity were collected, the protein content and the enzyme activity were determined and they were pooled. Enzyme was dialyzed overnight against two changes of 2 liters of buffer A.

The dialyzed sample was applied to the Concanavalin A-Sepharose 4B (40 ml) affinity column previously equilibrated with buffer A containing 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 M NaCl (buffer B). After applying the protein solution, the column was washed with the same buffer until the protein concentration dropped below 0.1 mg/ml. Enzyme was eluted by 1 M methyl-α-mannopyranoside dissolved in buffer B. Fractions (1.1 ml) containing SSAO activity were pooled and dialyzed overnight with two changes of 2 liters of buffer A.

Electrophoretic analyses

PAGE and SDS-PAGE analyses of the crude homogenate and the chromatographic fractions were determined according to the method of Laemmli (1970) under reducing and non-reducing conditions. Gels were stained in 0.1% Coomassie Blue R-250 and also silver-stained (Bio-Rad).

Determination of SSAO activity

SSAO activity was measured according to the method of Tabor et al. (1954) when benzylamine (BA) was used as substrate. The molar extinction coefficient of benzaldehyde was taken as $11{,}800\ M^{-1}\ cm^{-1}$.

Estimation of molecular weight

The approximate molecular weights of the SSAO samples were determined by SDS-PAGE.

Synthesis of the novel pyrazoline derivatives

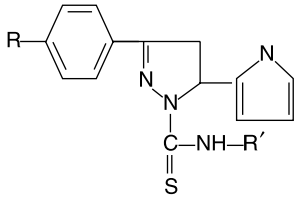
Twelve 1-N-substituted thiocarbamoyl-3-substituted phenyl-5-(2-pyrolyl)-2-pyrazoline derivatives were synthesized in the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hacettepe University by methods previously described (unpublished data).

Analysis of kinetic data

Newly synthesized compounds 3a–3l were dissolved in dimethyl sulfoxide (DMSO) and used in the concentration range of 5–1000 μM. Inhibitors were incubated with purified SSAO at 37°C for 0–60 min prior to addition to the assay mixture. Possible MAO activity was inhibited by preincubating the enzyme with l-deprenyl (1 μM) for 60 min at 37°C. Reversibility of the inhibition of SSAO by these compounds was assessed by dilution. Kinetic data for interaction of SSAO with these compounds were determined using Microsoft Excel package program. IC_{50} values were determined from plots of residual activity percentage, calculated in relation to a sample of the enzyme treated under the same conditions without inhibitors, versus inhibitor concentration [I].

Protein determination

Protein contents of the samples were determined according to the method of Bradford (1976).



3a–3l

Comp.	R	R′	Comp.	R	R′
3a	$-CH_3$	$-CH_3$	3g	–Cl	$-CH_3$
3b	$-CH_3$	$-CH_2CH_3$	3h	–Cl	$-CH_2CH_3$
3c	$-CH_3$	$-CH_2CH{=}CH_2$	3i	–Cl	$-CH_2CH{=}CH_2$
3d	$-CH_3$	$-C_6H_5$	3j	–Cl	$-C_6H_5$
3e	$-OCH_3$	$-CH_3$	3k	$-OCH_3$	$-CH_2CH{=}CH_2$
3f	$-OCH_3$	$-CH_2CH_3$	3l	$-OCH_3$	$-C_6H_5$

Scheme 1

Results and discussion

Purification of SSAO

Two chromatographic steps (Cibacron Blue 3GA-agarose column and Concanavalin A-Sepharose 4B affinity column) (Figs. 1 and 2) were used to purify SSAO from rat lung. The purity of the enzyme was estimated to be about 46-fold greater than that of the crude extract, with an overall yield of about 15%. The specific activity of the final enzyme preparation was 5.554 nmol/min/mg of protein under standard assay conditions.

Molecular characteristics of the purified SSAO

Polyacrylamide gel electrophoresis (PAGE) under non-denaturating conditions gave a single band which corresponded to a molecular mass of 184 kDa, when stained with Coomassie Blue R-250. SDS-PAGE analysis of the purified enzyme also revealed a single band with apparent molecular weight of 184 kDa. However, when the solubilized and purified enzyme samples were treated with

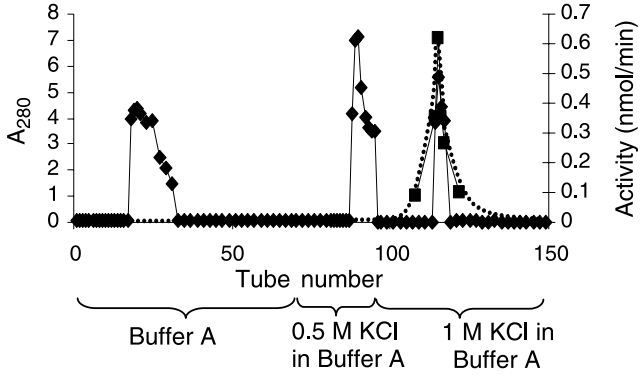


Fig. 1. Elution profile of rat lung SSAO from Cibacron Blue 3GA-agarose column. 0.8 ml fractions were collected with a flow rate of 5 ml per h. (-◆-) A_{280}, (··■··) Enzyme activity. Activity was determined by using benzylamine as substrate and expressed as nmol/min. Buffer changes are depicted by braces

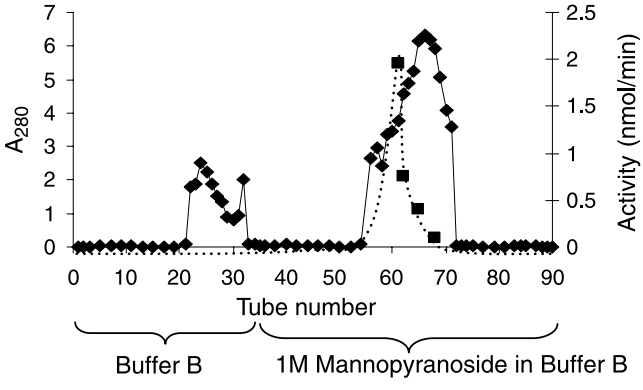


Fig. 2. Elution profile of rat lung SSAO from Concanavalin A-Sepharose 4B affinity column. 1.1 ml fractions were collected with a flow rate of 6 ml per h. (-◆-) A_{280}, (··■··) Enzyme activity. Activity was determined by using benzylamine as substrate and expressed as nmol/min. Buffer changes are depicted by braces

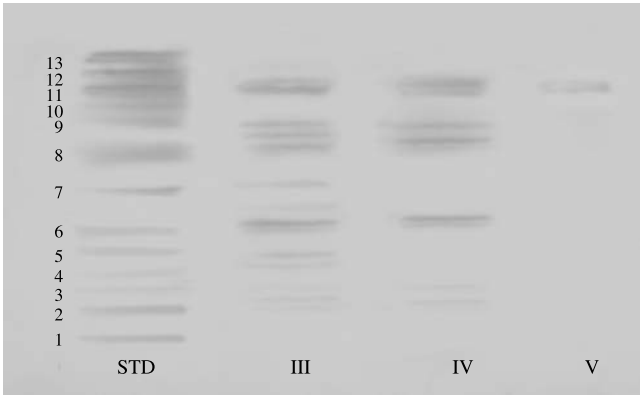


Fig. 3. SDS-PAGE (7.5%) pattern of the solubilized and purified rat lung SSAO under reducing conditions. The gel was silver-stained for protein. Lane *STD* corresponds to the molecular weights of known proteins whereas the remaining lanes correspond to the solubilized SSAO (*III*); SSAO eluted from Cibacron Blue 3GA-agarose column (*IV*) and SSAO eluted from Concanavalin A-Sepharose 4B column (*V*). In Lane STD: *1* aprotinin 6.5 kDa; *2* α-lactalbumin 14.2 kDa; *3* trypsin inhibitor 20 kDa; *4* rypsinogen 24 kDa; *5* carbonic anhydrase 29 kDa; *6* glyceraldehyde-3-phosphate dehydrogenase 36 kDa; *7* ovalbumin 45 kDa; *8* glutamic dehydrogenase 55 kDa; *9* albumin 66 kDa; *10* fructose-6-phosphate kinase 84 kDa; *11* phosphorylase b 97 kDa; *12* β-galactosidase 116 kDa; *13* myosine 205 kDa

Table 1. *IC_{50} values for the inhibition of rat lung SSAO by the novel compounds**

Compounds	IC_{50} for SSAO (μM)**	
	Preincubation 0 min	Preincubation 60 min
3a	NI	NI
3b	NI	NI
3c	NI	NI
3d	NI	NI
3e	70.11 ± 6.34	42.10 ± 4.26
3f	230.57 ± 19.50	170.16 ± 13.90
3g	NI	NI
3h	NI	NI
3i	NI	NI
3j	NI	NI
3k	280.30 ± 20.31	225.30 ± 16.44
3l	NI	NI
Semicarbazide	12.82 ± 1.20	5.40 ± 0.46

* Homogenates were preincubated with l-deprenyl (1 μM) at 37°C for 30 min to inhibit MAO-B activity. SSAO activity was determined at the 0 and 60 min preincubation of homogenates at 37°C, in the presence of different concentrations of the novel compounds and the remaining activity was measured by using benzylamine as substrate.

** Each value represents the mean ± SD of three independent experiments. *NI* No inhibition.

β-mercaptoethanol (2-ME), some of the bands collapsed to give faster bands in SDS-PAGE and the pure enzyme revealed a single broad band with an apparent molecular mass of 93 kDa. Similar bands were observed in SDS-PAGE pattern of the solubilized and purified samples of SSAO when the gel was silver stained (Fig. 3). According to this pattern rat lung SSAO may be a homodimer composed of 93 kDa subunits possibly attached by disulfide bridges.

Inhibition of the purified SSAO by novel pyrazoline derivatives

Compounds 3e, 3f and 3k, carrying a *p*-methoxy group on their phenyl ring, inhibited rat lung SSAO irreversibly in a time-dependent manner while the other compounds did not inhibit the enzyme (Table 1). K_i values of 43.01 ± 3.22, 66.27 ± 5.50, and $69.82 \pm 5.29\,\mu M$ were calculated for compounds 3e, 3f and 3k, respectively. It seems that the presence of an electron-rich substituent such as a methoxy group may be responsible for the inhibitory actions of these compounds. Compound 3e showed the highest inhibitory potency towards SSAO. However, elongation of the side chain at the R′ position was found to decrease the SSAO inhibitory activity, and the presence of an aromatic ring at the R′ position caused the complete loss of the inhibitory activity of the compound. It is suggested that these compounds cannot enter the first small active site cavity of the enzyme and may interact tightly with some other reactive groups present in the molecule. It was previously proposed that compounds in which the apparently non-competitive and irreversible SSAO-inhibitory activity increases with increasing time period are called suicide inhibitors (Kinemuchi et al., 2000; Lyles, 1995; Lizcano et al., 1994, 1996; Holt and Callingham, 1995). Since with-

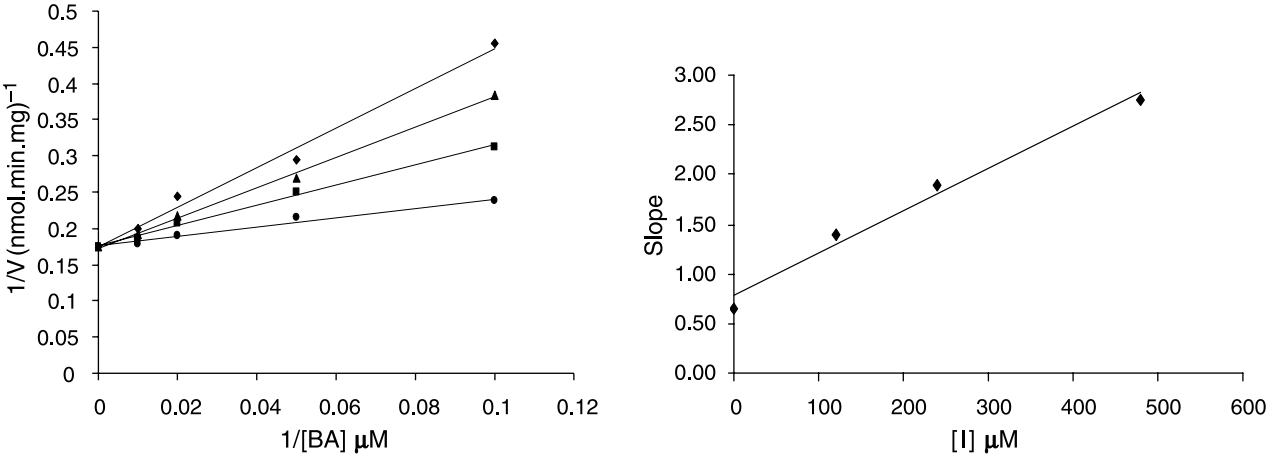


Fig. 4. Lineweaver-Burk plot corresponding to the inhibition of rat lung SSAO with compound 3e without preincubation. Benzylamine was used as substrate in the concentration range of 10–100 μM. [3e] = (-●-) 0 μM, (-■-) 120 μM, (-▲-) 240 μM and (-◆-) 480 μM

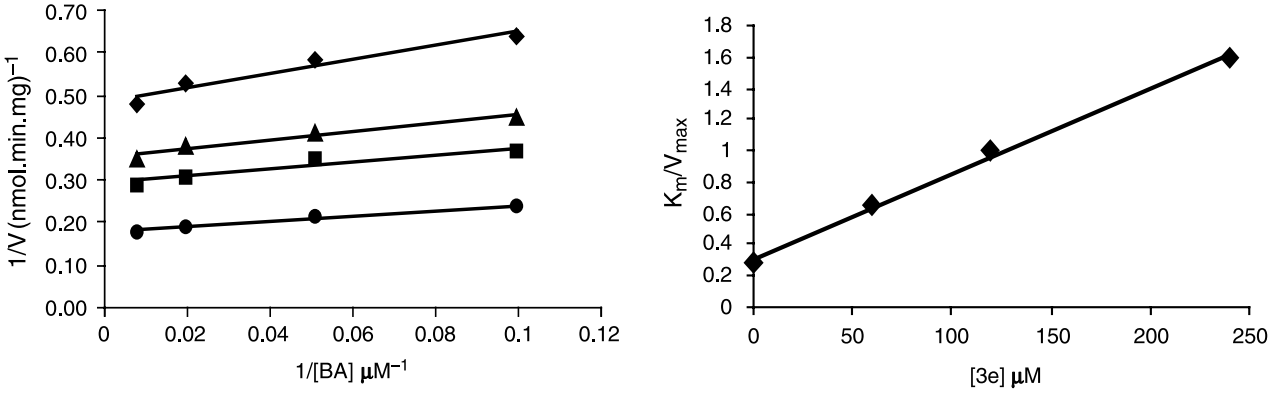


Fig. 5. Lineweaver-Burk plot corresponding to the inhibition of rat lung SSAO by compound 3e with preincubation. Benzylamine was used as substrate in the concentration range of 10–100 μM. SSAO was incubated with the compound at 37°C for 60 min prior to adding to the assay mixture [3e] = (-●-) 0 μM, (-■-) 60 μM, (-▲-) 120 μM and (-◆-) 240 μM

out preincubation the mode of inhibition with compound 3e was competitive with K_i value of 58.5 μM (Fig. 4), whereas after 1 h of preincubation the mode of inhibition changed to non-competitive with K_i value of 43.01 μM (Fig. 5), it is suggested that these derivatives behave as suicide inhibitors of rat lung SSAO. The presence of a thiosemicarbazide group in the parent pyrazoline molecule may be responsible for the interaction of these derivatives with SSAO.

In conclusion, compounds 3e, 3f and 3k may be used to discriminate between Cu- and FAD-containing amine oxidases and to determine the possible roles of SSAO in physiological events and also in some SSAO-related disorders. Furthermore, it can be concluded that these compounds may have promising features as anti-Parkinson agents if their SSAO-inhibitory effects can be supported by *in vivo* studies.

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